TNFα induced IKKβ complex activation influences epithelial, but not stromal cell survival in endometriosis

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Running title; IKKβ in endometriosis

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Abstract;

Study Hypothesis: Can the activity of the IκB kinase (IKKβ) complex in endometriotic cells contribute to endometriotic lesion survival?

Study answer: There is a constitutive activity of the IKKβ catalytic complex in peritoneal and deeply infiltrating lesions that can influence epithelial, but not stromal cell viability.

What is known already: Endometriotic lesions exist in an inflammatory microenvironment with higher local concentrations of cytokines, such as tumor necrosis factor α (TNFα). TNFα stimulates the activation of the IKKβ complex, an important nodal point in multiple signalling pathways that influence gene transcription, proliferation and apoptosis. However, few data on the regulation of IKKβ in endometriotic tissue are currently available.

Study Design, size, duration: A retrospective analysis of endometriotic tissue from peritoneal, ovarian and deeply infiltrating lesions from 37 women.

Participants/materials, setting, methods: Basal and activated (phosphorylated) IKKβ concentrations were analysed by Western blotting and immunohistochemistry. The relationship between the expression and activation of these proteins and peritoneal fluid (TNFα) concentrations, measured via ELISA, was examined. A subsequent *in vitro* analysis of TNFα treatment on the activation of IKKβ and the effect on epithelial and stromal cell viability by its inhibition with PS1145 was also performed.

Main results and role of chance; Levels of the phosphorylated IKKβ complex in endometriotic lesions had a significant positive correlation with peritoneal fluid TNFα concentrations. Phosphorylated IKKβ complex was more prevalent in peritoneal and DIE lesions compared to ovarian lesions. IKKβ was present in both epithelial and stromal cells in all lesions but active IKKβ was limited to epithelial cells. TNFα stimulated an increased expression of phosphorylated IKKβ and the inhibition of this kinase with PS1145 significantly influenced ectopic epithelial cells viability but not eutopic epithelial cells, or endometrial stromal cells.

Limitations, reasons for caution; *In vitro* analysis on epithelial cells was performed with immortalized cell lines and not primary cell cultures and only low sample numbers were available for the study.

Wider implications of the findings; The regulation of aberrant signalling pathways represents a promising yet relatively unexplored area of endometriosis progression. The IKKβ complex is activated by inflammation and is critical nodal point of numerous downstream kinase-signalling pathways, including NFκB, mTOR and BAD. This study shows a significant relationship between peritoneal fluid TNFα and IKKβ activation in epithelial cells that will have significant consequences for the continued survival of these cells at ectopic locations through the regulation of downstream pathways.

Large scale data: none
Study funding/competing interests; The study was funded by the Swiss National Science Foundation (Grant Number. 320030_140774). The authors have no conflict of interest to declare.

Key Words; endometriosis, kinase, signalling, IKK, TNF, inflammation, transcription factor, DIE, peritoneal, endometrioma

Introduction

Endometriosis is characterized by the growth of endometrial epithelial and stromal cells outside the uterine cavity. It is an extremely prevalent disease occurring in 10-20 % of women of reproductive age and is accompanied by chronic pelvic pain and subfertility. Although the exact pathogenesis is not yet clear Sampson's theory of transplantation is commonly accepted (Sampson, 1928). This theory proposes that viable endometrial epithelial and stromal cells are refluxed back through the Fallopian tube into the peritoneal cavity during menstruation. Once in this ectopic environment these cells avoid immune detection, invade the underlying mesothelial layer and stimulate a chronic inflammatory response.

Numerous inflammatory cytokines and chemokines (Borrelli *et al.*, 2013; 2014) are increased in the peritoneal fluid of women with endometriosis, which occurs through the coordinated interaction of the refluxed endometrial and peritoneal immune cells. Refluxed endometrial cells produce and secrete chemokines (Hornung *et al.*, 1997) that attract leukocytes and activated peritoneal macrophages (Halme *et al.*, 1983). The activated macrophages produce inflammatory cytokines, which in turn further stimulate cytokine production by the endometrial cells, creating a feed forward regulatory loop (Lebovic *et al.*, 2001) and the chronic inflammatory environment. This inflammatory environment has the potential to both influence symptomology (McKinnon *et al.*, 2015) and disease progression (Bruner-Tran *et al.*, 2013).

A chronic inflammatory environment can contribute to endometriotic lesion progression through the activation of a series of intracellular kinase signalling pathways (McKinnon *et al.*, 2016). The IκB kinase (IKK) complex represents a significant, early nodal point in many of the kinase signalling pathways. In the nuclear factor (NF)κB signalling pathway the IKK complex removes the inhibitory IκB protein from NFκB allowing translocation into the nucleus and gene transcription (Bonizzi and Karin, 2004) subsequently influencing the gene expression of many cytokine and chemokines, immunoreceptors, cell adhesion molecules, stress response genes, and growth factors (Pahl, 1999). IKKβ also interacts with the tubular sclerosis (TSC2) protein in the mammalian target of rapamycin (mTOR) pathway influencing cellular proliferation (Lee *et al.*, 2007) and phosphorylates Bcl2-antagonist of cell death (BAD) pathway suppressing apoptosis (Yan *et al.*, 2013).

The IKKβ complex consists of two catalytic subunits, IKKα and IKKβ and one regulatory subunit (IKKγ) (Hinz and Scheidereit, 2014). The binding of extracellular tumor necrosis factor (TNF)α to its cell membrane receptor TNFR (Haider and Knöfler, 2009) stimulates the phosphorylation of both IKKα and IKKβ and activation of the IKKβ complex. TNFα

concentrations are increased in the peritoneal fluid of endometriosis (Harada *et al.*, 1999) and are correlated with the severity of the disease (Bedaiwy *et al.*, 2002) and thus TNFα stimulated IKKβ activity may have a significant influence on the endometriotic lesions.

At present very little information is available on the expression and activity of this important upstream nodal kinase in endometriotic cells. In this study we used a combination of clinical and *in vitro* experiments to determine the presence and importance of IKKβ in endometriosis. We found that peritoneal fluid TNFα concentrations had a significant positive correlation with the activated IKKβ complex and that this was most likely due to epithelial cell expression. Furthermore IKKβ activity was important in regulating ectopic epithelial cell, but not stromal cell survival. These results therefore suggest a significant role of IKKβ in endometriotic epithelial cells that deserves further attention.

Methods;

Patient samples

Prior to surgery the relevant institutional review board granted ethical approval and informed consent was obtained from all patients. During surgery performed for suspected endometriosis samples of endometrium, peritoneal fluid and endometriotic lesions were collected. Endometrial biopsies were collected via soft curette (Pipelle de Cornier, Laboratoire CCD, France) and stored in RNAlater (Thermo Fischer Scientific, USA) at -80 °C as described previously (Santi *et al.*, 2011). Peritoneal fluid was collected during the laparoscopic procedure from the cul-de-sac and centrifuged to remove blood cells, aggregates and debris. Samples were excluded if blood remained in the samples. The pelvic cavity was examined and any endometriotic lesions were removed and the patient staged (no endometriosis, or stage I-IV) according to the revised American Fertility Society staging system (rAFS) ("Revised American Society for Reproductive Medicine classification of endometriosis," 1997). The lesions were recorded as either peritoneal, ovarian or deeply infiltrating endometriosis (DIE). All surgeries were performed during the proliferative phase of the menstrual cycle and endometriosis was confirmed by histological analysis.

Endometrial biopsies were collected from both women with (n = 8) and without endometriosis (n = 7) and used for the isolation of primary cells via collagenase digestion and size exclusion, as described previously (McKinnon *et al.*, 2012). Strong yields were obtained for the primary stromal cells (ESC) from all women except one without endometriosis, however only limited amounts of epithelial cells could be successfully isolated. As endometrial epithelial cells are terminally differentiated and do not propagate immortalized epithelial cells were acquired. Matching peritoneal fluid of sufficient quality was not always available for cytokine measurement therefore if peritoneal fluid was available the ectopic lesions were immediately frozen and stored for stored fresh frozen for subsequent Western blot analysis. If peritoneal fluid was not available, they were formalin fixed and paraffin embedded for immunohistochemistry analysis.

Cytokine measurement in peritoneal fluid

TNFα was measured by an enzyme-linked immunoabsorbent assay (ELISA) kit (R&D Systems, Abingdon, England) using a high-sensitivity NADH cascade amplified alkaline phosphatase with antigen-antibody incubations at 28 °C in a dry incubator and at a dilution of 1:2 in the diluent provided. Peritoneal fluid progesterone concentrations were also measured via a radioimmunoassay (Coata-count, DPC; Buhlmann Laboratories, Allschwil, Switzerland) to confirm the patient cycle phase (McKinnon *et al.*, 2014).

Protein isolation and Quantification in ectopic lesions

Approximately 30mg of fresh frozen ectopic tissue was used to prepare whole cell extracts via homogenization with the FastPrep 120 tissue homogenizer (30 seconds at 4.0 m/s) in radioimmunoprecipitation assay buffer (RIPA; 50mM Tris-Cl, pH 7.4, 150mM NaCl, 1mM EDTA, 1% v/v triton X-100, 1 % w/v sodium deoxycholate, 0.1% w/v sodium dodecyl sulfate and 1% v/v protease and phosphatase inhibitor cocktail (Cell Signalling Technology, Danvers, Massachusetts)). Final protein concentrations were determined by the bicinchoninic acid assay (QuantiPro BCA; Sigma).

Proteins separation was performed by heating 20µg of total protein into LDS buffer (Invitrogen) to 70 °C for 10 minutes and running it on a 4 -15 % Novex NuPAGE Bis/Tris gel (Invitrogen). Proteins were transferred to a 0.45µm nitrocellulose membrane in 4-morpholinepropanesulfonic acid buffer (MOPS; Invitrogen) pH 7.7 for 1 hour. Non-specific binding was blocked by incubation with 5% bovine serum albumin (BSA) in phosphate buffered saline (PBS) containing 0.1% Tween 20 (PBST). Membranes were probed with rabbit anti-IKKβ antibody (1:1000) (Cell Signalling Technology), and with rabbit anti-pIKKα/β(176/180)(1:1000) antibody (Cell Signaling Technology), or mouse anti-actinβ antibody (Abcam, Cambridge, UK) 1:5000. Secondary anti-rabbit (GE Healthcare, Opfikon, Switzerland) and anti-mouse (Sigma) antibodies conjugated to horseradish perioxidase were diluted 1:50 000 and 1:200 000 respectively. Immuno-reactivity was determined with the SuperSignal West Femto kit (Pierce; Thermo Scientific) using the Chemi-Doc XRS+ system (Bio-Rad Laboratories, AG, Cressier, Switzerland). Band densitometry was quantified with the Quantity One software and in each Western blot a calibrator sample with strong IKKβ, and pIKKα/β expression was included to normalize concentrations across gels and determine protein concentrations relative to the calibrator sample. The relative expression of each sample was expressed as a percentage of the calibrator. Actinβ was used as a loading control.

Immunohistochemistry

Immunohisotchemistry was performed using serial sections of 4µm mounted onto glass slides (Superfrost, Braunschweig, Germany), dewaxed in xylene and rehydrated through a series of decreasing ethanol concentrations. Epitope retrieval was performed with 10mM citrate buffer, pH 5.5 for 5 minutes in a 450W microwave. Endogenous perioxidase activity was blocked with 3% hydrogen perioxide (H_2O_2) and a blocking step performed with 3% BSA for 30 minutes in Tris buffered saline (TBS; Tris 100mM, NaCl 0.15 M; pH 7.4). Rabbit anti-IKKβ antibody (1:100) and rabbit anti-pIKKα/β (176/180) antibody (1:100) were diluted in 3% BSA in TBS and incubated at 4 °C overnight in a humidified chamber. Slides were washed with TBS and 0.1% Tween 20 (TBST) prior to incubation with an affinity purified, biotin

conjugated goat anti-rabbit antibody (Dako, Glastrup, Denmark) for 90 minutes at room temperature. After a final wash slides were incubated with an avidin-biotin HRP complex (Vectastain, ABC Kit, Vector Laboratories, Burlingham, CA, USA) for 45 minutes. The antigen-antibody complex was detected by incubation with 3,3' diaminobenzidine substrate and slides were counterstained with hematoxylin and mounted in Aquatex (Merck). The primary antibodies were excluded for the negative controls. Images were photographed with a Nikon Eclipse E800 microscope (Nikon, Japan). Semi-quantitative analysis of antibody staining in the epithelial and stromal cells of the endometriotic lesions was determined by the allocation of scores between 0-3; 0 (negative), 1 (weak), 2 (moderate) and 3 (strong), based on the intensity of staining. The percentage of cells with positive immuno-reactivity was also determined and allocated a score between 0-6 as described 0% = 0, 1-10% = 1, 11-30% = 2, 31-50% = 3, 51-70% = 4, 71-90% = 5; > 91% = 6 in each cell type of the endometriotic lesions. For the final immuno-reactive score the staining intensity and percentage of positive cells was multiplied, as described previously (Samartzis *et al.*, 2012).

Cell culture and TNFα treatment

Isolated primary endometrial stromal cells (ESC) were maintained in Iscoves's modified Eagle medium (IMEM) (Invitrogen) supplemented with 10% fetal calf serum (FCS) (Invitrogen) and 1% antibiotic/antimycotic (Invitrogen). The immortalized epithelial cell lines, EM E6/E7 and EM'osis, were provided by Professor Kyo, Kanazawa, Japan and were isolated from eutopic endometrium (Kyo *et al.*, 2003) and an ectopic endometrioma (Bono *et al.*, 2012) respectively. These cells were maintained in Dulbecco's modified Eagles medium (DMEM) (Invitrogen) with 10% FCS and 1% antibiotic/antimycotic. The 12Z cells were provided by Professor Starzinski-Powitz, Goethe University and were originally isolated from a peritoneal endometriotic lesion (Zeitvogel *et al.*, 2001). These cells were also maintained in complete DMEM media.

To determine the influence of TNFα on pIKKα/β activity in all cell types the cells were seeded into 6 well plates at approximately 3 $x10^5$ cells/ well. After reaching approximately 80% confluence the media was changed to 0.5% FCS for overnight incubation prior to treatment. Cells were treated either with control media (0.5% FCS in normal media) or control media plus 10ng/ml and 100ng/ml recombinant human TNFα (R&D systems, United Kingdom) for 6 hours. At the end of the treatment period the cells were rinsed and collected in RIPA buffer.

MTS assay and treatment with PS1145

PS1145 is a small molecular weight compound that is a specific inhibitor of IKKβ activity (Lam *et al.*, 2005). Inhibition of IKKβ activity with PS1145 was performed in 96 well plates

seeded at a density of $6x10^3$/ well. Twenty-four hours prior to treatment the cells were changed into serum free media and treatment media prepared by diluting PS1145 into either serum free media at a final concentration of 1μM. Subsequent concentrations were prepared by a 1:3 serial dilution (333.33nM, 111.11nM, 37.04nM, 12.3nM & 4.12nM). Cell viability was measured after 72 hours by the CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega). Triplicate wells were used for each cell type and experiment. For the immortalized epithelial cell lines the experiment was repeated three times and for the primary ESC an experiment on each of the eight endometriosis and seven non-endometriosis preparations was performed separately. A control (without PS1145) was included for each experiment and designated as 100% viability and subsequent values expressed as a percent of control.

Statistical Analysis;

All statistical analyses were performed with Graphpad Prism version 6.0. The correlation between the peritoneal fluid cytokines and IKKβ and pIKKα/β expression was performed using the non-parametric Spearman's rank correlation coefficient. Two groups comparisons were performed with a non-parametric Mann-Whitney U test and the comparison of three or more groups with the non-parametric Kruskal-Wallis One-way analysis (ANOVA) and Dunn's multiple comparison *Post hoc* test. The interaction between two variables was determined via a two-way ANOVA with a *post-hoc* Tukey's multiple comparison test to determine the difference between individual groups or conditions.

Results;

Patient data and characteristics

In total, endometriotic tissue was removed from 37 endometriosis patients and endometrial biopsies from eight women without endometriosis. Of these 37 women we collected accompanying peritoneal fluid from 21 in order to compare peritoneal fluid TNFα and ectopic IKKβ. Of the 21 samples 14 were collected from women without any hormonal treatment, four were using combined oral contraceptives (COC) and three were using GnRH analogues. No significant variation in TNFα, IKKβ or pIKKα/β based on hormonal use (**Table 1**) was identified. Five of the lesions were peritoneal, eleven ovarian and five DIE.

The remaining 16 samples without accompanying peritoneal fluid were kept for immunohistochemistry. Of these, six women had no history of hormonal treatment, five were using COC and five were using GnRH analogues. Three lesions were peritoneal, seven ovarian and six DIE. No significant variation was observed between either pIKKα/β, or IKKβ expression in both the epithelial and stromal cells based on hormonal treatment (**Table II**).

Brett McKinnon 28.7.2016 22:48
Kommentar [1]: The value in the table has been corrected

IKKβ expression and activation in endometriotic tissue and its relationship to peritoneal fluid TNFα concentrations*;*

Comparison of peritoneal fluid TNFα and endometriotic lesion IKKβ and pIKKα/β showed a significant positive correlation between TNFα and pIKKα/β ($r = 0.6268$, $n = 21$, $p = 0.0024$) (**Figure 1A**), but not IKKβ ($r = 0.4216$, $n = 21$, p 0.0570) (**Figure 1B**), as determined by semi quantitative Western blot (**Figure 1C**). A significant variation in pIKKα/β concentrations between lesions from different locations ($p < 0.05$) was observed with a *post-hoc* analysis confirming a significantly lower expression in ovarian lesions (50 ± 8.7, $n = 11$) compared to the peritoneal lesions (99 ± 9.7, $n = 5$) ($p = 0.041$) (**Figure 1D**). No significant difference was observed with the DIE lesions (91 ± 21.6, $n = 5$). In contrast, IKKβ expression showed no variation between lesion types ($p = 0.4905$) with similar expression in the peritoneal (138 ± 29.3, $n = 5$), ovarian (128 ± 26.7, $n = 11$) and DIE lesions (80 ± 26.9, $n = 5$) (**Figure 1E**). The active to inactive ratio of IKKβ (pIKKα/β:IKKβ) varied significantly between lesions ($p = 0.0123$) with both peritoneal (1.0 ± 0.39, $n = 5$) and DIE (1.7 ± 0.54, $n = 5$) higher than ovarian lesions (0.5 ± 0.09, $n = 11$) with a *post-hoc* analysis showing a significant difference between DIE and ovarian lesions ($p = 0.0168$).

Cell specific activation of IKK in endometriotic lesions

Immunohistochemistry identified a low but uniform expression of IKKβ in peritoneal (**Figure 2A**), ovarian (**Figure 2B**) and DIE lesions (**Figure 2C**) in both stromal (*red arrows*) and epithelial cells (*black arrows*). In contrast, pIKKα/β in peritoneal (**Figure 2D**), ovarian

(**Figure 2E**) and DIE (**Figure 2F**) lesions was predominantly epithelial (*black arrows*), with significantly less stromal cells expression (*red arrows*). Negative controls showed no expression in peritoneal (**Figure 2G**), ovarian (**Figure 2H**), or DIE (**Figure 2I**) lesions. No statistically significant difference was observed in IKKβ expression between either lesion location ($p = 0.2420$) or cell type ($p = 0.1972$) (**Figure 2J**), although this could be due to a lack of power. pIKKα/β expression was significantly different in cell types ($p = 0.0198$), but no statistically significant difference could be observed between lesion type ($p = 0.3402$) (**Figure 2K**)possibly again due to lack of power.

Chris Ford 27.7.2016 11:38
Kommentar [2]: Please be wary of interpreting this to mean that the values are similar.

Brett McKinnon 28.7.2016 23:00
Kommentar [3]: I have included the phrase 'statistically significant'

IKKβ activity after TNFα treatment

Western blot analysis of pIKKα/β after TNFα treatment of epithelial cell cultures confirmed a low but positive expression in all cell lines examined **(Figure 3A).** Semi-quantitation of band densitometry indicated that the 12Z cells showed the strongest basal expression (no TNFα), but this did not vary after TNFα treatment ($p = 0.2320$). The EM'E6/E7 cells showed lower basal expression and also no significant variation after TNFα treatment ($p = 0.4475$). In contrast, TNFα treatment of EM'osis cells significantly increased pIKKα/β above the no treatment control (46 ± 5.0, $n = 3$) at concentrations of both 10ng/ml (141 ± 34.2, $n = 3$, $p = 0.0173$) and 100ng/ml (197 ± 23.3, $n = 3$, $p = 0.0085$) (**Figure 3B**). Similar Western blots were performed on protein lysate isolated from stromal cells however no protein expression could be observed in these preparations.

Cell specific influence of IKKβ inhibition on viability

In the epithelial cell cultures there was a significant influence of PS1145 on cell viability based on cell type ($p < 0.0001$) (**Figure 4A**). No significant effect of PS1145 was observed on the EM E6/E7 at any concentration. For EM'osis cells there was a significant increase in cell viability at the lowest concentrations (PS1145 2.43nM; 123 ± 0.2, $n = 3$, $p < 0.001$) that was gradually diminished as concentrations increased (PS1145 1μm; 106 ± 6.3 $p > 0.05$), whereas 12Z cell viability was significantly decreased at the lowest concentrations (PS1145 2.43nM; 70 ± 4.6, $n = 3$, $p < 0.0001$) and remained significantly reduced through to the highest concentration (PS1145 1μm; 73 ± 2.6, $n = 3$, $P < 0.0001$). In contrast primary ESC isolated from women with and without endometriosis showed no significant variation based on either PS1145 ($p = 0.8868$) or cell type ($p = 0.3516$) (**Figure 4B**).

Discussion

In this study we examined the expression of the IKKβ protein kinase complex and the activation of its catalytic subunits pIKKα/β in both endometriotic lesions, as well as its influence on cell survival in *in vitro* models. The results show a significant relationship between the phosphorylation of the IKKβ complex and peritoneal fluid TNFα. Subsequent immunohistochemistry staining showed that although no statistically significant difference in IKKβ expression was observed across all cells and lesion types the phosphorylated IKKβ complex was predominantly epithelial. This data was supported by the *in vitro* studies that confirmed pIKKα/β expression in epithelial cell culture models, but not primary stromal cells and that inhibition of IKKβ activity significantly influenced endometriotic epithelial cell viability, but not eutopic epithelial cell viability, nor the viability of endometrial stromal cells from women with and without endometriosis. These results therefore suggest that a dysregulation of the IKKβ kinase occurs in ectopic epithelial cells that may be related to the inflammatory microenvironment. Given the role of IKKβ in transmitting extracellular signals into cell survival via kinase signalling pathways it may represent a significant molecule in endometriosis pathogenesis.

At present there is very little known about the role of IKKβ in endometriosis. The results from our clinical samples suggest that the constitutive IKKβ activity is significantly different between peritoneal and DIE lesions compared to ovarian lesions. This difference of expression was supported by our *in vitro* results that showed TNFα stimulated an increase in pIKKα/β expression and that inhibition of IKKβ activity increased the Em'osis cell viability. In contrast in the peritoneal derived epithelia cells TNFα had a limited influence of pIKKα/β expression and inhibition of IKKβ activity decreased cell viability. Unfortunately a DIE derived cell line was not available.

Endometriosis is a significantly heterogeneous condition, although whether these lesions have different pathologies (Nisolle and Donnez, 1997), or represent a continuum of the same disease (Somigliana *et al.*, 2004) is still debated. These data suggest a varied cellular response to inflammation may occur in different lesions. It has previously been shown that rectovaginal septum lesions have a distinctly inflammatory phenotype (Bertschi *et al.*, 2013) and that concentrations of inflammatory mediators are stronger in the peritoneal fluid of DIE compared to lesions from other locations (Santulli *et al.*, 2012). As IKKβ can be associated with TNFα both in ours and other studies (Lee *et al.*, 2007) it is possible that the increased production of inflammation associated with DIE lesions is related to the higher IKKβ activity.

Chris Ford 27.7.2016 11:50
Kommentar [4]: Please consider if this conclusion is justified. Some groups are small so statistical power is limited.

Brett McKinnon 28.7.2016 23:00
Kommentar [5]: I have changed the wording to temper the conclusion

The identification of a TNFα influenced activation of the IKKβ in endometriotic tissue is significant because of the multiple downstream pathways it regulates **(Figure 5)** and the influence this can have on gene transcription, protein translation and both cellular proliferation and apoptosis. Activation of IKKβ stimulates NFκB gene transcription and a constitutive activation of NFκB has been observed in peritoneal endometriosis (González-Ramos *et al.*, 2007). Multiple factors present in the peritoneal fluid of women with endometriosis including cytokines and iron overload (Alvarado-Díaz *et al.*, 2015) may lead to this constitutive activation. Furthermore an increased NFκB activity has been linked to recurrence of ovarian endometrioma (Shen *et al.*, 2008). Neither of these studies however examined IKKβ expression directly. In the immortalized epithelial 12Z cells IKKβ inhibition attenuated inflammatory cytokine secretion (Grund *et al.*, 2008) and in ectopic endometrial stromal cells miR200a suppresses IKKβ (Dai *et al.*, 2012), raising the possibility of suppressed IKKβ activity in stromal cells occurs via an epigenetic regulation. In contrast to its role in inflammation via the NFκB pathway, IKKβ can also regulate cellular proliferation and apoptosis through the mTOR and BAD pathways (Dunlop and Tee, 2014) (Yan *et al.*, 2013). A dysregulation of mTOR has previously been implicated in endometriosis pathogenesis of DIE lesions leading to increased proliferation (Leconte *et al.*, 2011), as has a role for mTOR mediated autophagy (Choi *et al.*, 2014) and BAD activation in ovarian endometriomas (Stickles *et al.*, 2015). Together this suggests that TNFα has the potential to modulate all of these activities via IKKβ activation.

We found that the constitutive activation and influence on cell survival was largely restricted to epithelial cells. Endometriotic lesions are a combination of epithelial and stromal cells and an interdependency between the cells types is required for endometriotic lesions to continue proliferating as tissue integrity of refluxed endometrial matter is essential to endometrial tissue implantation (Nap *et al.*, 2003). We have also previously shown that the stromal cells produce significantly more inflammatory cytokines than epithelial cells in response to stimulation (Bersinger *et al.*, 2008). It could therefore be postulated that a paracrine regulation occurs in the lesions through the stromal cells production of cytokines stimulating a constitutive activation of the IKKβ complex in epithelial cells, which ultimately contributes to cell survival. More research however is required to explore this hypothesis.

Furthermore, the activity of IKKβ in other cell types other than endometriotic cells was not directly addressed in this study, but may give further insight into this mechanism in normal tissue. For endometriosis, however whether this mechanism also happens in healthy eutopic epithelial cells may be of less consequence. This is because epithelial cells will only be present in the peritoneal cavity when endometriosis is present, and when endometriosis is

present there is a constant inflammatory environment. We believe it is this confluence of ectopic epithelial cells and constant inflammation that makes the contribution of TNFα stimulated IKKβ activity significant. It may be such that this is a characteristic not inherent in the endometrium, but rather acquired during the life of the lesion and contribute more to progression through a constant stimulation of the inflammatory cascade. Further study on whether there is a significant difference between the activation of IKKβ in the eutopic endometrium of women with and without endometriosis would be an interesting follow-up.

Whether other cell types also show a constitutive activity of IKKβ in the presence of inflammation would also be interesting. In this study the images in Figure 2 indicate cells proximal to the endometriotic lesion are largely negative for pIKKα/β expression, providing circumstantial evidence for the preferential activation of pIKKα/β in endometriotic epithelial cells. Previous studies suggest that cells proximal to the lesion may have different characteristics to cells distal to the lesions (Young *et al.*, 2014) and these cells thus may also be interesting to study, however we were unable to collect this tissue due to our current ethical approvals. Future studies on this topic may however be warranted.

Limitations of this study were the inclusion of women with hormonal treatment. Previous research however has suggested there was no significant influence on NFκB activation by oral contraceptives (González-Ramos *et al.*, 2007). We also observed no statistically significant difference for IKKβ in this study, although the power of this analysis was limited by the small sample size. It is possible that this may introduce a variability in peritoneal fluid cytokine concentrations in endometriotic women, as GnRHa analogues have been shown to have an influence on the inflammatory environment (Nirgianakis *et al.*, 2013), however we postulate that a reduction in inflammatory cytokines by hormonal treatment would also be reflected by a subsequent reduction in IKKβ activation, maintaining any correlation between the extra and intracellular environment. A further limitation of this study is the small sample size. An expansion of the sample number would provide more definitive information on both the influence of TNFα, as well as hormonal treatments, on IKKβ activity. It is difficult to draw direct conclusions on the contribution of IKKβ to cell survival in different lesions types as immortalized cell lines were used, however given the similarity observed in the *in vitro* results to the clinical samples we can be confident that IKKβ has a role in epithelial endometriotic cells.

Chris Ford 27.7.2016 12:03
Kommentar [6]: Given the number of observations and the variance, please consider whether hormonal; effects can be excluded with such confidence.

In conclusion we have observed a significant relationship between TNFα and the activation of IKKβ complex in the endometriotic microenvironment and that this activation occurs predominantly in the epithelial cells of peritoneal and DIE lesions. Furthermore IKKβ

435 inhibition *in vitro* significantly influenced epithelial cell, but not stromal cell behaviour. The
436 regulation of kinase signalling pathways is a significant, but under explored area of
437 endometriosis pathogenesis and progression and may represent potential non-hormonal
438 treatment targets for endometriosis (McKinnon *et al.*, 2016). Given the ability of the
439 extracellular inflammatory environment to influence IKKβ activity and its subsequent affect
440 on downstream pathways this kinase may be significant interest in endometriosis.
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Authors Roles; **VK** performed experiments, cell culture and assisted with sample collection. **CW** performed immunohistochemistry and analysis. **GG** assisted with cell culture. **NAB** assisted with sample collection and intellectual development of the project. **MDM** assisted with sample collection and intellectual development of the project. **BDM** conceived project, performed experiments and prepared the manuscript.

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Conflict of Interest:

None

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Table 1; Comparison of endometriotic lesion and peritoneal fluid protein expression in samples removed from women using different hormonal treatment. Data are Mean ± SEM

	Hormone treatment				
	No hormone (n=14)	Combined oral contraceptive (n = 4)	GnRH analogue (n=3)	Total (n =21)	p
pIKKα/β (%)	66 ± 9.8	77 ± 27.8	92 ± 17.5	72 ± 8.5	0.4732
IKKβ (%)	120 ± 23.5	90 ±27.7	151 ± 27.7	119 ± 16.9	0.3819
TNFα (pg/ml)	2.1 ± 0.61	1.2 ± 0.53	1.5 ± 0.54	1.9± 0.43	0.7721

-IKKβ and pIKKα/β protein values were determined via Semi-quantitative Western blot densitometry analysis and expressed as a percentage of a standardised control sample included in each Western blot, as described in detail in the methods section.

-TNFα values represent peritoneal fluid TNFα concentrations determined by ELISA and expressed as pg/ml.

-Analysis of significance was performed via the non-parametric One-way ANOVA test (Kruskal-Wallis) and a post hoc comparison of all groups (Dunn's multiple comparison test) significance $p < .05$

Table II: Comparison of protein expression in formalin fixed paraffin embedded endometriotic tissue according to hormonal treatment. Data are mean ± SEM

Hormonal use	No hormone (n= 6)	Combined oral contraceptive (n = 5)	GnRH analogue (n=5)	Total (n =16)	*P
pIKKα/β					
Epithelial	1.7 ± 0.56	3.4 ± 1.78	6.2 ± 2.27	3.6 ± 0.98	0.108
Stromal	0.3 ± 0.33	0.8 ± 0.37	0.6 ± 0.60	0.5 ± 0.24	0.448
IKKβ					
Epithelial	2.3 ± 0.95	2.2 ± 1.11	3.0 ± 1.76	2.5 ± 0.70	0.949
Stromal	0.7 ± 0.42	12.4 ± 1.75	0.0 ± 0.00	1.00 ± 0.58	0.327

-Values represent the scores derived from the semi-quantitative IHC analysis, described in detail in the methods.

-Analysis of significance was performed via the non-parametric One-way ANOVA test (Kruskal-Wallis) and a post hoc comparison of all groups (Dunn's multiple comparison test) significance $p < .05$

Brett McKinnon 7.2.2017 09:39

Kommentar [7]: A typo resulted in an additional 1 at the start of the number. The correct value is 2.4

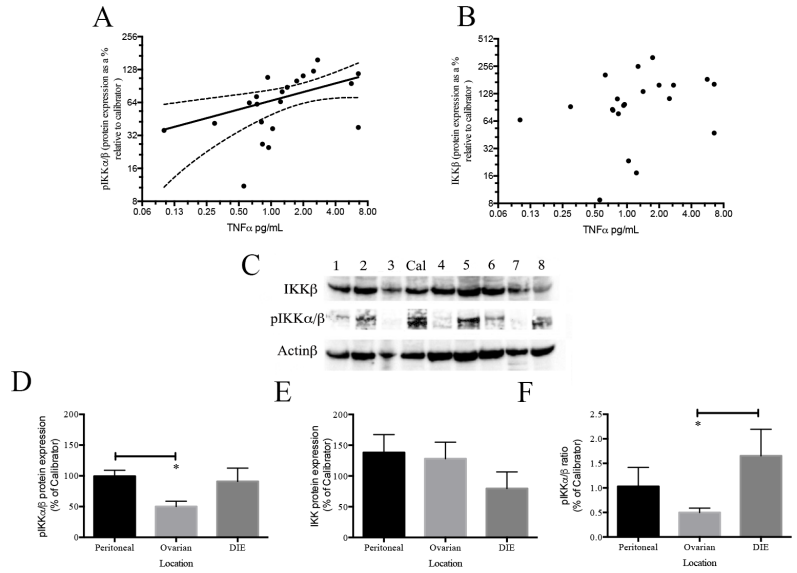

Figure 1; IKKβ activation in endometriosis and its relationship to tumor necrosis factor (TNF)α. (A) A significant correlation (r = 0.6268, n = 21, p = 0.0024) was present between the peritoneal fluid TNFα expression and the pIKKα/β(176/180) protein complex in endometriotic lesions. **(B)** No significant association was observed between the expression of endometriotic lesion IKKβ and peritoneal fluid TNFα expression. **(C)** Western blot analysis of endometriotic tissue samples confirmed a consistent presence of both IKKβ. In contrast the expression of pIKKα/β varied significantly amongst samples. Actinβ was used as a loading control. **(D)** Analysis of pIKKα/β expression indicated that high concentrations were identified in the peritoneal and DIE lesions with lower concentrations observed in the ovarian lesions. **(E)** Basal IKK was more uniform amongst all samples, although with a slightly lower, but non-significant expression in the DIE lesions. **(F)** Analysis of the pIKKα/β: IKKβ ratio confirmed a lower ratio of activation in the ovarian lesions that was significantly lower than that observed in DIE lesions. Protein concentration in all components was calculated as relative to the calibrator sample and expressed as a percentage. Correlation was determined performed by Spearman's Rank correlation coefficient and comparison between lesion location performed by a non-parametric Kruskal-Wallis One-Way anaylsis (ANOVA) test with a *post-hoc* Dunn's multiple comparison. * $p < 0.05$. (P) peritoneal, (O) ovarian and DIE (deeply infiltrating endometriosis.

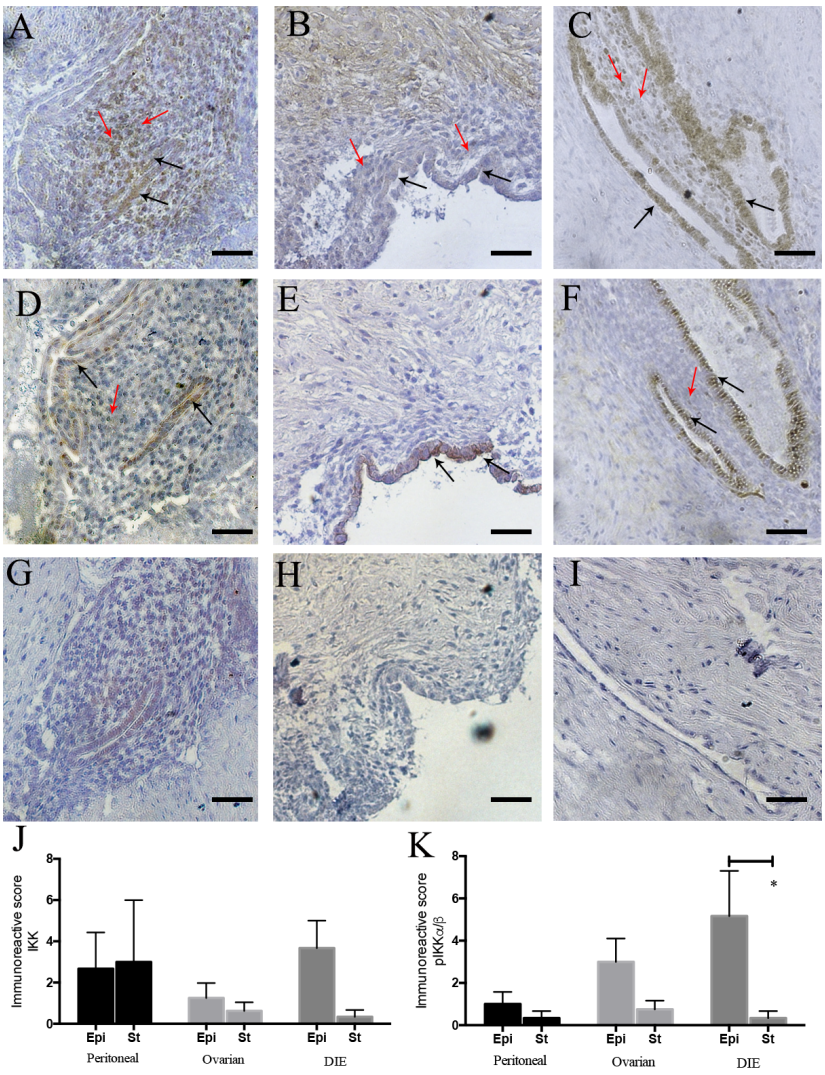

Figure 2; Cell specific expression and activation of IKKβ in endometriotic lesions. Basal IKKβ expression was observed in both epithelial (*black arrows*) and stromal cells (*red arrows*) of endometriotic lesions removed from the **(A)** peritoneal, **(B)** ovarian, and **(C)** DIE regions. The expression of the activated IKK complex (pIKKα/β) was limited predominantly to the epithelial cells (*black arrows*), although some stromal cell expression was observed (*red arrows*). This was consistent across lesions from the **(D)** peritoneal, **(E)** ovarian and **(F)** and DIE lesions. Negative controls showed no expression in lesions from the **(G)** peritoneal **(H)** ovarian, or **(I)** DIE region. A semi-quantitative analysis of the cell specific expression indicated that no statistically significant variation in **(J)** IKKβ expression was observed between epithelial and stromal cells, however pIKKα/β was significantly stronger in the epithelial cells for all lesions with a largest difference observed in the DIE lesions. **(K)** Analysis of protein activation and expression between cell type and lesion location was performed with a Two-way analysis of variance (ANOVA) test with a *post-hoc* Tukey's multiple comparison. Scale bars = 50μm. * $p < 0.05$

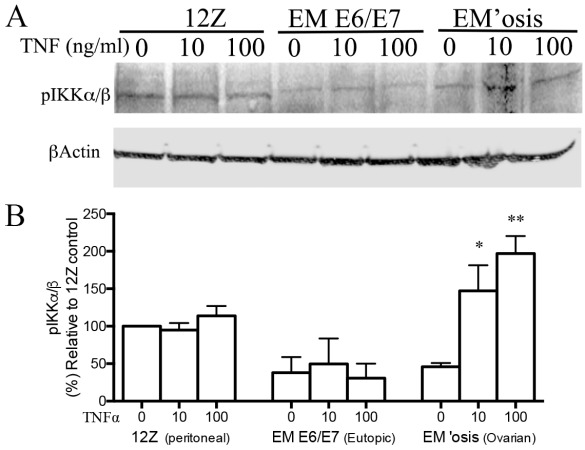

Figure 3; pIKKα/β after tumor necrosis factor (TNF)α treatment in endometriotic epithelial cells. (A) Western blot analysis confirmed the expression of pIKKα/β in the 12Z, EM E6/E7 and EM 'osis cell lines both with and without TNFα treatment. **(B)** Semi-quantitative analysis indicated TNFα did not significantly influence either 12Z, or EM E6/E7 expression. There was however a significant increase in pIKKα/β after both 10ng/ml and 100ng/ml TNFα in the EM'osis cell line.

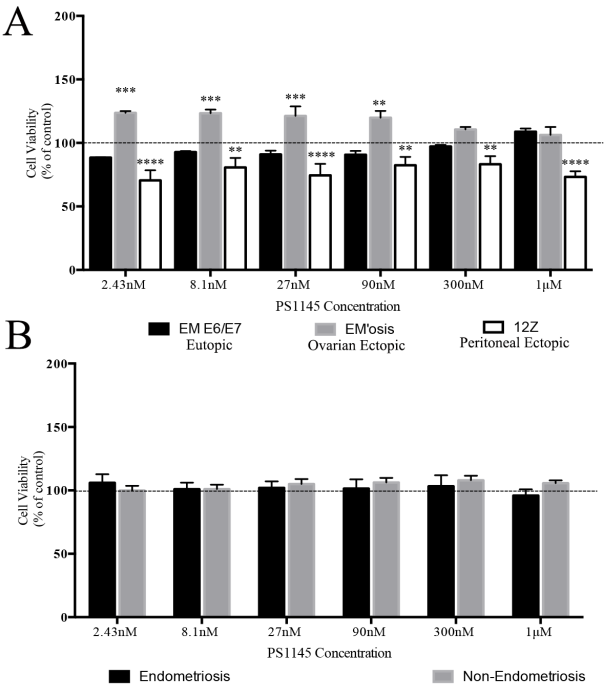

Figure 4; Influence of IKKβ inhibition on epithelial and stromal cell viability. (A) Inhibition of IKKβ activity with increasing concentrations of PS1145 had no influence on the eutopic derived epithelial EM E6'E7 cells, significantly increased the cell viability of the ovarian derived EM'osis cells, significantly reduced the viability of the peritoneal derived 12Z cells. **(B)** PS1145 had no influence on stromal cells from women with and without endometriosis. Analysis on the influence between cell type and PS1145 concentrations on cell viability performed with a Two-way analysis of variance (ANOVA) test with a post-hoc Tukey's multiple comparison test. ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

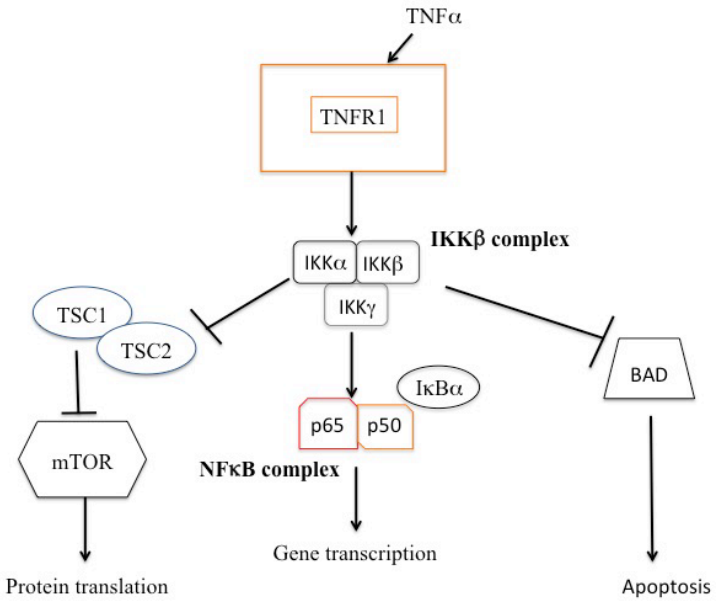

Figure 5; The IKK complex and its influence on downstream signalling pathways. Binding of extracellular tumor necrosis factor (TNF)α to TNF receptor 1 (TNFR1) stimulates the phosphorylation and activation of IKKα and IKKβ that exist as a complex along with IKKγ. Activation of IKKβ leads to the phosphorylation of IκBα, which under basal conditions is bound to the p65 subunit of the NFκB complex. Phosphorylation of IκBα removes it from the NFκB complex and initiates protesomal degradation allowing NFκB translocation into the nucleus and gene transcription. Activation of the IKK complex can also lead to an interaction with the tuberous sclerosis (TSC)2 protein that exists in a heterodimer with TSC1. Inhibition of TSC2 activity increases the activity of the mammalian target of rapamycin (mTOR) complex stimulating both protein translation and cellular proliferation. Activation of the IKK complex also leads to an inactivation of the BH3 only BAD protein inactivating TNFα stimulated apoptosis.